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Diverse origins of tetracycline resistance in the honey bee bacterial pathogen *Paenibacillus larvae*

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Abstract

Paenibacillus larvae is the causative agent of the important honey bee larval disease American Foulbrood (AFB). This pathogen has been treated in bee colonies by a single registered antibiotic, oxytetracycline (OTC), for fifty years. Recently, widespread resistance to OTC has been reported. In this study, the degree of antibiotic resistance was contrasted with DNA sequence variation for 125 *P. larvae* isolates collected in North America. Resistance was uncorrelated with bacterial haplotype, suggesting either that resistance has evolved multiple times in *P. larvae* or that resistance involves recent horizontal transfer via a non-genomic (e.g., plasmid or conjugal transposon) route. The recency of OTC resistance in *P. larvae* across this broad survey area underscores the need to manage foulbrood infections carefully and to monitor populations for resistance.

Keywords: *Apis mellifera*; Pollination; Pathogen; Plasmid; tetA

1. Introduction

Honey bees, *Apis mellifera*, play an important agricultural role worldwide (Morse and Calderone, 2000) and are important pollinators in many natural ecosystems. Domesticated and feral honey bees suffer from numerous parasites and pathogens, including mites, beetles, fungi, protozoa, viruses, and bacteria (Morse and Flottum, 1997). *Paenibacillus larvae* (Heyndrickx et al., 1996) is the primary bacterial pathogen in honey bees, and is the cause of the widespread disease American Foulbrood (AFB); (Shimanuki, 1997). Remarkably, the antibiotic oxytetracycline (OTC, marketed as Terramycin) has provided effective control of AFB for several decades. The longevity of OTC as a control probably results in part from the severe handling of most outbreaks of foulbrood disease in bee colonies. Thanks to past damages from this disease, AFB is monitored closely by governmental inspection agencies and suspected outbreaks in North America and elsewhere generally lead to the incineration of both bees and equipment (Shimanuki, 1997).

Despite these measures, OTC resistance in *P. larvae* has become widespread in the past decade (e.g., Miyagi et al., 2000), motivating new searches for alternate antibiotics (Kochansky et al., 2001) and for honey bee genetic stock that is better able to fend off disease (Spivak and Reuter, 1998). Another key to the management of this bacterium is to better understand the mechanisms of OTC resistance and its spread in *P. larvae* populations. Knowing how resistance is achieved in *P. larvae*, as in other bacteria (Roberts, 1996), should help predict the efficacy of new, alternative, antibiotics (Kochansky et al., 2001). If resistance has arisen multiple times, it will be helpful to know if this was achieved through similar or completely independent mechanisms. It will also be of interest to determine whether this agricultural pathogen shows routes to resistance that are similar to those of human disease pathogens, given concerns over agricultural antibiotic use and cross-resistance in human pathogens (Smith et al., 2002).

There are several outstanding questions related to the spread of resistance in *P. larvae*. First, do geographically distant populations of OTC-resistant bacteria represent

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independent origins of this trait, or a single origin followed by dispersal? The former has implications for management strategies to minimize the evolution of new resistant bacterial strains while the latter has implications for the transport of bees and hive materials, a billion-dollar industry in the US alone (Morse and Calderone, 2000). Also, can genetic markers be used to identify resistant strains of *P. larvae*? If so, tests using these markers offer promise as a diagnostic tool and these markers themselves may speed the identification of mechanisms behind OTC resistance. Finally, why did OTC resistance in *P. larvae* become apparent only recently (Alippi, 1996; Miyagi et al., 2000) despite long-term use of this antibiotic? Specifically, are there signs that this resistance has been present, but unnoticed, for a longer period of time in some populations?

Here, I combine laboratory assays of antibiotic resistance with DNA sequence data to search for genetic correlates between resistant isolates of *P. larvae*. A total of 125 North American isolates of *P. larvae* were collected and assayed for both resistance and sequence variation for the 16S rDNA gene (Ash et al., 1991; Govan et al., 1999). Previous population-level studies in *P. larvae* have successfully exploited variation at randomly amplified genomic regions (specifically BOX and ERIC primer sites; Alippi and Aguilar, 1998a,b) but these markers have not been compared with resistance. Standard biochemical traits in *P. larvae* were shown to be unlinked with OTC resistance by (Alippi, 1996).

The isolates analyzed here include both present-day samples and samples that predate any records of antibiotic resistance. While both resistance to tetracycline and bacterial haplotypes varied substantially within and across populations, there was no strong relationship between specific bacterial haplotypes and antibiotic resistance.

2. Materials and methods

Isolates of *P. larvae* were sampled from those received by our Bee Disease Diagnostic Service from May-July, 2001. These isolates were derived from infected bees and hive materials collected by apiary inspectors and beekeepers in Alberta, Canada ($n = 32$ collections), New York State, US ($n = 34$), South Dakota, US ($n = 33$), and 12 additional North American states and provinces ($n = 25$). In addition, four historical samples (pre-dating any records of OTC resistance) were included, along with three South American (Chilean) isolates used as outgroups.

Spore suspensions of bacterial isolates were used to inoculate brain-heart infusion agar plates (Difco) fortified with 0.1 mg/L thiamine hydrochloride (pH 6.6). A single paper disk impregnated with 5 μg tetracycline (BBL) was placed in the center of each plate. After 72 h of incubation (34 °C) plates were scored for growth by measuring the diameter of the zone of inhibition with respect to the tetracycline disk. Among those cultures that showed growth characteristic of *P. larvae* (Knox and Shimanuki, 1997), resistant isolates were defined as those for which the zone of inhibition around the tetracycline disk was less than 15 mm in radius.

Bacteria were taken directly from media plates, using sterile cotton swabs. The tips of these swabs were then immersed in 500 μl of a 5% Chelex-100 (Bio-Rad) solution. After soaking for 5–10 min, swabs were stirred and removed from the Chelex solution. The solution was incubated at 95 °C for 5 min, then was pelleted by centrifugation. A 708 bp fraction of the 16S ribosomal RNA gene was amplified from aliquots of this the supernatant (approx. 50 ng DNA) using the polymerase chain reaction (PCR) and oligonucleotide primers FB16S1S (5-TGG GGAGCAAACAGGATTAG-3′) and FB16S1A (5′-AC GGGCGGTGTGTACAAG-3′), designed from the published sequence of this gene (position 774–1412; Ash et al., 1991).

PCR products were verified by agarose-gel electrophoresis prior to purification. Oligonucleotide primers were removed by exonuclease digestion followed by ethanol cleanup and precipitation. The 16S rDNA gene fragment was sequenced in both directions, using primers FB16S1S and FB16S1A and a cycle-sequencing protocol that relied on the end-terminator Big Dye 2.0 (Applied Biosystems). Sequencing reactions were analyzed by capillary electrophoresis and fluorescence scoring on an ABI 3700 sequencing machine (Applied Biosystems).

DNA sequences for the 125 isolates, along with the three Chilean samples and the published sequence for this region (GenBank No. X60619) were aligned using the Sequencher software program (version 4.1, GeneCodes). This alignment showed no insertion–deletion events among the AFB samples, for a 556-base region within the amplified gene. Samples were compared by neighbor joining (Swofford, 1999) using the phylogenetic analysis program PAUP 4.03b (Sinauer Associates).

3. Results

3.1. Haplotypic variation

Four 16S rDNA haplotypes, arbitrarily labeled A, B, C, and D (GenBank Accession Nos. AY260765–AY260768), were present in 125 North American samples assayed here. These haplotypes differed by 1–5 nucleotides from each other, and by 2–6 nucleotides from the South American outgroup sample (which was identical to the sequence described as *P. larvae larvae* by Ash et al., 1991). Populations differed significantly in the relative frequency of these four haplotypes (G test,

Table 1
Paenibacillus larvae haplotype and collection area with susceptibility to tetracycline

Haplotype	New York		S. Dakota		Alberta		Others	
	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
A	0	8	2	5	14	11	0	8
B	4	15	11	10	1	4	1	14
C	0	4	1	2	–	–	0	2
D	0	3	1	1	3	0	–	–

$\chi^2 = 34.3, \mathrm{df} = 9, p < 0.0001$). Most strikingly, samples collected in Alberta, Canada, showed a high frequency of haplotype A (25/33, 70%), while haplotype B was most common in all populations sampled in the US (Table 1). In total, these two haplotypes were present in 86% of all isolates.

3.2. Tetracycline resistance

Based on zones of inhibition around the tetracycline disk, these samples showed a clear split between resistant and susceptible strains (Fig. 1). Fifty one of 176 (29%) isolates were classified as being resistant to tetracycline. None of the historical samples showed signs of resistance, a result consistent with previous screenings (Shimanuki and Knox, 1994). Samples from different sites differed significantly in degree of resistance. Specifically, samples from Alberta showed high levels of resistance (27/51 = 57%) compared to other populations. This result could reflect sampling biases in different areas, however, and further collections are needed.

There was no correlation between resistance and haplotype in the samples as a whole (Fig. 2). Resistant and susceptible isolates occurred within each of the four observed haplotypes. When analyzed separately by population, only the Alberta samples showed a slight correlation between haplotype and resistance (G test, $\chi^2 = 6.2, \mathrm{df} = 30, p < 0.05$). Even in these Alberta samples, each of three haplotypes present had both resistant and susceptible members. Further, while isolates with haplotype A showed high levels of resistance in the Alberta samples, those with haplotype B were most frequently resistant in each of the other populations (Table 1).

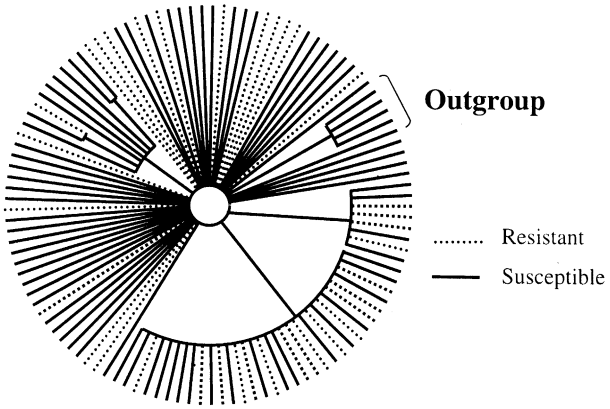

Fig. 2. Phylogenetic tree showing widespread resistant and susceptible isolates in four haplotypes. Outgroup, samples of *P. larvae* from Chile and original isolate from Ash et al. (1991).

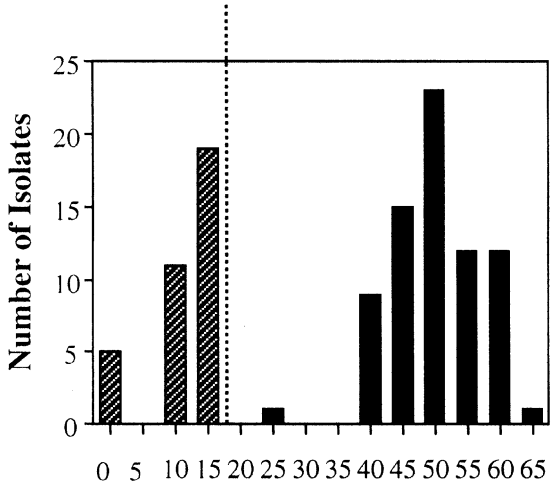

Fig. 1. Zones of inhibition (mm) of *P. larvae* isolates exposed to disk containing 5 μg tetracycline. Dotted line shows defined cut-off between resistant and susceptible isolates.

4. Discussion

Tetracycline resistance in North American populations of *P. larvae* has become widespread only in the past decade, despite long-term use of this antibiotic. Given this recency, it is tempting to assume a single origin of tetracycline resistance, followed by dissemination of this trait through the movement of honey bees and equipment or hive products. The genetic results described here discount this scenario. Instead, tetracycline resistance from widespread areas appears to reflect independent origins of this trait over a short time period. Alternatively, tetracycline resistance might be epigenetic in nature, specifically through the presence of plasmids and mobile genetic entities that produce proteins involved in resistance (Adams et al., 1998). If so, there may be only minimal linkage between bacterial haplotype and the degree of resistance.

It was hoped that genetic markers derived from this study could be used to predict the risk of resistance in specific isolates, thereby improving the management of this pathogen, through both more assiduous removal of infected materials and bees and the use of alternate controls. The results are discouraging for the use of such markers, although haplotype differences in specific populations (e.g., Alberta) may have predictive power for resistance. There remains a need to find markers specifically tied to resistance mechanisms, as has been done in other environmental pathogens (Teo et al., 2002). Should the resistance be plasmid- or transposon-mediated, there is some promise in identifying conserved genes tied to resistance by these mechanisms. For example, the TetA gene, a major source of efflux-based resistance carried by the conjugal transposon TN10, is highly conserved at the DNA sequence level (Teo et al., 2002). Plasmid genes conferring resistance (e.g., for the human pathogen *Aeromonas salmonicida*; Adams et al., 1998) are similarly conserved between diverse bacterial hosts. Even gene-specific markers may prove ineffective if the types of resistance mechanisms are diverse. In the absence of markers that encompass the major resistance mechanisms, laboratory assays of individual isolates remain the preferred route to determine levels of resistance for disease management.

Searches for the mechanism behind tetracycline resistance will benefit from known mechanisms of resistance to this antibiotic and its relatives (as discussed in Kochansky et al., 2001). A directed, genetic, approach to finding the mechanism seems more viable than a marker-based strategy. Specific candidate genes, including Tet genes and transposable elements (Roberts, 1996), could be screened for both activity level in response to tetracycline, and for genetic variation between resistant and susceptible isolates.

The environmental causes behind the recent arrival of tetracycline resistance remain open for speculation. Resistance is unlikely to have arisen because of a change in regulation of this disease, since official management policies toward this pathogen have remained unchanged for decades (Shimanuki, 1997). The appearance of tetracycline resistance does mirror one major change faced by honey bee colonies in North America. An unprecedented number of new pests have arrived in North American honey bee colonies over the past decade. It is possible that these other agents have weakened honey bee colonies to the extent that greater levels of antibiotics are required to maintain them. Higher, more prolonged, antibiotic use might have favored the emergence of resistant strains. It is further possible that colonies weakened by other pests and pathogens are acting as larger reservoirs for *P. larvae* in honey bee populations, again increasing the probability that resistant strains will evolve and persist.

The results underscore the need to review and enforce honey bee pathogen management procedures. While ensuring that significant numbers of bees are not lost to this important disease, control methods should always consider the risk of generating populations of resistant bacteria. These methods should incorporate both accurate molecular assays for *P. larvae* in bees and hive products (Govan et al., 1999) and in vitro assays of pathogen type and resistance (Knox and Shimanuki, 1997). Breeding strategies aimed at developing bees with both hygienic behavior (Spivak and Reuter, 1998) and larval resistance to this disease also will be key. Finally, as new antibiotics become available for the treatment of this disease (Kochansky et al., 2001), strict guidelines should be established for the rotation of different antibiotic treatments, to prolong their effectiveness as controls. These methods should be integrated with those controlling other honey bee pests and pathogens to minimize the long-term cost and effort of maintaining healthy, vigorous colonies.

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